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TITLE OF THE THESIS : **To assess the potential anti-viral activity of Rosamarinic Acid and Leutin against 4 serotypes of Dengue Virus**

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ABSTRACT

OBJECTIVE: The study was performed to evaluate *in silico* binding ability of Leutin/Lutein and Rosamarinic acid/Rosmarinic acid (RA) with the envelope domain III (EDIII) proteins of the four serotypes of dengue virus (DENV). Also, *in vitro* cell-free interaction with EDIII protein of each serotype and the inhibiting ability using a functional plaque assay enlightening potential antiviral activity of the two compounds.

METHODS AND RESULTS: EDIII protein structures for the four DENV serotypes were retrieved from Protein Data Bank (PDB) and used as receptors. Four ligands of Lutein and four of RA were selected from the ZINC database and used for computational molecular docking and ligand interaction analysis with the four receptors using bioinformatics tools like AutoDockVina and Molecular Operating Environment (MOE) software. The EDIII of the four serotypes demonstrated significant interaction, RA ligand ZINC899870 particularly presented best binding energy values of -6.4, -7.0, and -6.9 kcal/mol with EDIII of serotype DENV-1, DENV-2, and DENV-4 respectively. From the results predicted by MOE, the hydroxyl (OH) of 3, 4-dihydroxyphenyl group of RA ligand ZINC899870 is actively involved in interaction with all four serotypes. However, when the two compounds were screened for the ADME properties by subjecting to the Lipinski rule of five, Lutein did not clear the filter test and hence was eliminated from further *in vitro* studies. Using a bio-layer interferometry (BLI) based platform, the interaction kinetics of RA to DENV-EDIII proteins of the four serotypes demonstrate interaction which can be arranged in an increasing order of magnitude EDIII-DENV1 > EDIII-DENV2 > EDIII-DENV3 > EDIII-DENV4. Subsequently, the inhibiting ability of RA using plaque assay indicating the ability of RA not only to bind, but also inhibit the four serotypes in cell culture, while being non-toxic at the concentrations used in the study. However, the effect of

RA was variable on different serotypes, demonstrating highest effect on serotypes DENV1 and DENV2 followed by DENV3 and DENV4.

CONCLUSION: RA is a competent candidate for further evaluation of potential antiviral activity that can be effective in conferring protection against the four serotypes of DENV.

INTRODUCTION

One of the primary constraint or challenge in developing an effective dengue vaccine has been the presence of the multiple serotypes of DENV (DENV1, DENV2, DENV3 and DENV4) (1,15). A critical and essential criterion that limits effective monoclonal antibody therapy and vaccine development is that the candidate should confer effective protection against all the four serotypes of the virus, lacking any cross reactivity. Failing to do so could contribute to a phenomenon known as antibody dependant enhancement (ADE), caused by heterotypic antibodies against diverse serotypes (16,17). Therefore, there is a need for identification of agents or molecules capable of impeding virus propagation. A drug can be considered a potential antiviral agent if it acts on any stage of the virus infection or replication cycle, being non-toxic to the body's own cells (18). The possible mode of action of antiviral agents would include being able to incapacitate extracellular virus particles by preventing its attachment to the target cells for infection. DENV targets the cells of immune system belonging to lymphoid lineage and keratinocytes. The E protein of flaviviruses is the one most widely studied owing to its critical role in viral attachment and penetration in the host cells. E protein consists of three distinct domains (domain I, domain II and domain III). Envelope domain III (EDIII) is known to participate in receptor recognition and binding (9). Therefore, blocking EDIII could theoretically restrict the virus from receptor recognition and entering the host cell. The drug under

consideration should have wide spectrum effect against maximum serotypes of the virus to be considered as a potent anti-dengue viral drug.

Phytochemicals have for a long time been the source of many a drug molecules indicated in a wide array of conditions (10–12). Lutein is a naturally occurring carotenoid, which is known for it's immunomodulatory and antioxidant activities. The activity of the full-length promoter (Fp) of the hepatitis B virus (HBV) transcription has been previously shown to be inhibited by Lutein (19). Rosmarinic Acid (RA) is a caffeic acid ester found in a variety of plants and is well known for antioxidant and anti-microbial activities. It was identified to be the principle molecule responsible for the antiviral activity of lemon balm in treating Herpes Simplex Virus (HSV) (20). Also, significant decrease in viral loads and proinflammatory cytokine levels in murine model of Japanese Encephalitis Virus (JEV) suggests its strong therapeutic effects against JEV (21), a flavivirus, closely related to the DENV.

Therefore, the current study was conducted to assess the *in silico* binding ability of Lutein and RA to the EDIII proteins of the four DENV serotypes. Using computational molecular docking analysis and ligand interaction study with the four receptors, we determine the related binding energy of interaction between the compounds and the EDIII proteins. The putative *in vitro* antiviral activity was evaluated using two approaches: a cell free drug target binding assessment to evaluate the binding ability of RA to envelope domain III (EDIII) protein of each serotype using a bio-layer interferometry (BLI) based platform and the inhibiting ability of RA by a functional plaque assay on Vero cells.

RATIONALE

Dengue fever is caused by DENV having four serotypes DENV-1, DENV-2, DENV-3, and DENV-4 that produce similar clinical presentations being genetically distinct (1–3). The antibodies generated against one serotype will confer lifelong protection against that particular serotype, while may cross react with a different serotype in a subsequent secondary infection. The cross reactivity may result in a phenomenon known as antibody dependent enhancement (ADE), leading to vigorous immune response (4–6). We do not have any specific antiviral drug treatment and the vaccine candidates have failed to show respectable proficiency against all the four serotypes (7,8). Probable alternative is to identify therapeutic agents, drug molecules which could impede or reduce virus titre. EDIII of DENV is involved in cognate host cell receptor recognition and adhesion (9). Therefore, blocking EDIII could theoretically restrict the virus from receptor recognition and entering the host cell. The drug under consideration should have wide spectrum effect against maximum serotypes of the virus to be considered as a potent anti-Dengue viral drug. Phytochemicals have for a long time been the source of many a drug molecule indicated in a wide array of conditions (10–12). The present study was designed to evaluate the therapeutic ability of two phytochemicals, lutein and rosmarinic acid (RA) against the EDIII proteins of the four DENV serotypes, enlightening the potential antiviral activity of the two compounds. They are well known for their anti-inflammatory and immune modulatory ability apart from the antiviral characteristics as demonstrated by several previous studies on related viruses (13,14).

AIM AND OBJECTIVES

Aim: To assess the potential antiviral activity of Rosamarinic Acid (RA) and Lutein against 4 serotypes of Dengue Virus (DENV).

Objectives

1. To assess the *in silico* interactions between RA and Lutein with the domain III envelope protein (EDIII) of the DENV serotypes 1-4.
2. To assess the *in vitro* cytotoxicity of RA and Lutein on suitable cell lines.
3. To assess the *in vitro* antiviral activity by using the plaque assay/qRT-PCR.
4. To calculate the Selectivity Index (SI) of the molecules as potential anti-DENV agents.

MATERIALS AND METHODS

Preparation of receptor

The structures for domain proteins of DENV with PDB ID: 3IRC, 2JSF, 3VTT and 2H0P corresponding to EDIII of DENV-1, DENV-2, DENV-3, and DENV-4 respectively. The receptor structures were modelled using structure modelling software PHYRE2. Using the UCSF Chimera tool, the structural anomalies were corrected in the side chain and folds and the structure was energy minimized.

Preparation of ligands

The ZINC database was explored for ligands of Lutein and RA, subsequently four ligands of Lutein ZINC40164432, ZINC14879961, ZINC14879959, ZINC8221225 and four ligands for RA ZINC5784598, ZINC13341234, ZINC00899870, ZINC00901160 were retrieved based on availability in the database. Structural modelling was performed using tools like Marvin Sketch, UCSF chimera and OpenBabel GUI.

Prediction of pharmacological property

Ligands were screened with ADME constraints according to Lipinski's rule of five and oral bioavailability to test the theoretical pharmacokinetics and predict the drug-likeness of the ligands. Using online prediction platform Molsoft L.L.C, molecular descriptors for Lipinski's rule were analysed for Lutein and RA.

Prediction of toxicity

Toxicity predictions were done using the LAZAR software which detects mutagenic and/or carcinogenic properties on the basis on the similarities in functional groups present in the LAZAR database.

Sequence analysis and structural comparison between EDIII of four DENV serotypes

Sequence comparison was performed using CLUSTALW, which carries out multiple sequence alignment. Structural comparison was accomplished using PyMOL software.

Molecular Docking

In order to understand how these ligands bind to the receptors, docking studies were performed on EDIII of the four DENV serotypes and 8 compounds of Lutein and RA by (4 for Lutein and 4 for RA) using AutoDockVina and ligand interaction study using Molecular Operating Environment (MOE) software.

BLI measurements

BLI based Octet Red 96 system was employed to analyse the binding kinetics of EDIII protein of four DENV serotypes with RA. Super Streptavidin (SSA) biosensors were used to immobilize the biotinylated EDIII proteins of four serotypes keeping a threshold response of 1.2 nm in independent experiments. RA was used as an analyte at concentrations of 330 µg/ml (925.8 µM), 110 µg/ml (308.6 µM) and 37 µg/ml (102.9 µM). Association and dissociation of ligand-analyte

complex was analyzed from the sensograms to determine dissociation rate constant (K_{off}) for each ligand.

Cell line

The Vero cells were employed for virus propagation and performing plaque assay.

Cytotoxicity Assay

MTT assay was employed for determination of *in vitro* cytotoxicity (CC_{50}) of RA on Vero cells. Cytotoxicity of RA was established with 6 dilutions from 10000 µg/mL to 0.1 µg/mL in triplicate treatments. Log_{10} concentration of RA was plotted against the mean percent cytotoxicity using a non-linear regression on Graph Pad Prism 7.04 software.

Virus culture and titration

The serotypes were propagated in Vero cells and titre was calculated for each serotype using plaque assay in independent experiments. Briefly, a serial 10-fold dilution of the serotype (DENV1-DENV4) were added onto the monolayer in a 24 well plate. The plates were incubated for virus adsorption for 1 hr and 30 min and methyl cellulose was overlayed to immobilize the virus. The plates were further incubated at 34°C in 5% CO_2 environment for 5 days. The assay was terminated by aspiration of spent medium from the wells and immunostaining of the plaques. The titre was expressed in the form of log_{10} plaque forming units per mL (log_{10} PFUs/mL).

Antiviral assay (Plaque assay)

RA was evaluated for antiviral activity by virtue of reduction in the number of plaques in plaque assay. Co-treatment: The virus titre for each of the four serotypes was adjusted such that countable plaques are obtained per well with an MOI (Multiplicity of Infection) of approximately 0.0002. The virus serotypes were exposed to different concentrations of RA in a ratio of 1:1 (v/v) to acquire an effective concentration of RA from 2000 µg/mL to 7.81 µg/mL. The mixture was incubated for 1 hr at 34°C and added to a confluent 24 well plate seeded with Vero cells. Plaque assay procedure followed was same as that outlined in virus titration assay.

Statistical analysis

All assays were carried out in triplicates. For the cytotoxicity assay, median cytotoxic concentration (CC_{50}) was calculated by Graphpad prism 7.04 software using non-linear regression. For antiviral assay, the data was normalized to consider 100% value for virus controls following the plaque count and subsequently the median effective concentration (EC_{50}) was calculated by Graphpad prism 7.04 software using 3 parameter non-linear regression analyses. The SI (selectivity index) for potential antiviral activity was calculated ($SI= CC_{50}/EC_{50}$) for each serotype.

RESULTS

Preparation of receptor

The structures of EDIII proteins of DENV with PDB ID: 3IRC, 2JSF, 3VTT and 2H0P corresponding to EDIII of DENV-1, DENV-2, DENV-3, and DENV-4 respectively were retrieved from PDB and used for the modeling study.

Preparatory modifications were performed on the PBD structure entries for the EDIII proteins of four DENV serotypes the 3D structures. The modified structure so obtained were saved in .pdb format and employed as receptor for all further docking studies.

Sequence analysis and structural comparison between EDIII of four DENV serotypes

Multiple sequence alignment using CLUSTALW software revealed the conserved regions between the EDIII proteins of the four DENV serotypes. The alignment indicated 75 % region of the entire sequence to be highly conserved and showcasing similar properties, indicating the active sites of the EDIII proteins to be conserved. The dissimilar regions were towards the terminal regions of the proteins indicating no effect towards the function of the protein.

Structural comparison between EDIII of four DENV serotypes using PyMOL software

Structural comparison was accomplished using PyMOL software. The superimposed secondary structures of the EDIII proteins of four serotypes presented overlapping between the Beta sheets indicating conserved regions.

Preparation of ligands

Four ligands of Lutein and four ligands for RA were retrieved from the ZINC database.

	Ligand	Mol. Wt.	H-bond Acceptor	H-bond donor	*LogP*	Molar Refractivity
Lutein	ZINC 40164432	568	*0*	*2*	10.414	184.144
	ZINC14879961	568	*0*	*2*	10.414	184.144
	ZINC 14879959	568	*0*	*2*	10.414	184.144
	ZINC 822125	568	*0*	*2*	10.414	184.144
RA	ZINC 5784598	359	4	4	1.639	87.873
	ZINC 13341234	359	4	4	1.639	87.873
	ZINC 00899870	359	4	4	1.639	87.873
	ZINC 00901160	359	4	4	1.639	87.873

Ligands for RA were retrieved from the Zinc database

The *.pdb* file format of the modified 3D structures was save and employed for docking studies.

Prediction of pharmacological property

Ligands were screened for ADME properties according to Lipinski's rule of five and oral bioavailability using Molsoft L.L.C (Molecular properties and drug likeliness) software. Lutein did not follow few parameters of Lipinski rules, such as molecular weight must be < 500 Da, Log P < 5, refractivity molar range must be between 40–130. Also, the predicted drug likeliness model score was calculated to be -0.33 which is on the lower end of drug likeliness scale for a compound. Whereas, RA has successfully cleared the Lipinski rules with a predicted drug likeliness model score of 0.37 which is at the positive end of drug likeliness scale for a

compound. Thus, it can be concluded that RA ligands showed high probability of success for drug likeness of the two compounds.

Toxicity prediction

LAZAR software predicted all the ligands of Lutein and RA as noncarcinogenic and nonmutagenic, with a probability of upwards of 0.025 signifying reliable predictions (Table 16). The ligands could indicate promising anti-dengue candidates.

Molecular docking results– Autodoc Vina

Lutein ligands	**3IRC (DENV-1)**	**2JSF (DENV-2)**	**3VTT (DENV-3)**	**2H0P (DENV-4)**
ZINC14879959	-5.9	-6.1	-7.9	-6.2
ZINC40164432	-6.0	-6.3	-7.4	-6.1
ZINC8221225	-5.7	-5.6	-6.9	-5.1
ZINC14879961	-6.1	-6.1	-7.1	-5.8

Binding energy score for interaction of Lutein ligands with EDIII receptors of four DENV serotypes generated by AutoDock Vina

RA ligands	3IRC (DENV-1)	2JSF (DENV-2)	3VTT (DENV-3)	2H0P (DENV-4)
ZINC00899870	-6.4	-7.0	-6.5	-6.9
ZINC00901160	-5.6	-6.5	-6.7	-6.6
ZINC13341234	-5.0	-6.5	-6.1	-5.6
ZINC5784598	-6.1	-6.6	-6.3	-6.2

Binding energy score for interaction of RA ligands with EDIII receptors of four DENV serotypes generated by AutoDock Vina

The analysed results suggest that EDIII of all four serotypes showed significant interaction with ligands of Lutein and RA. RA ligand ZINC00899870 demonstrated best binding energy values with DENV-1, DENV-2, and DENV-4 receptors. The docking scores for DENV-1, DENV-2, and DENV-4 were -6.4, -7.0, and -6.9 kcal/mol, respectively. Whereas Lutein ligand ZINC14879959 showed best binding energy value of -7.9 kcal/mol for EDIII of serotype DENV-3. The top 3 docking scores were observed for RA ligand, indicating RA has better conformation to bind to all four DENV-EDIII proteins.

Prediction of ligand interaction by MOE software for best docked ligands of Lutein and RA against the EDIII receptors of four DENV serotypes

From the results predicted by MOE, hydroxyl group of the ligands play an important role in the interaction of the ligands to the proteins. The hydroxyl (OH) of 3, 4-dihydroxyphenyl group of RA ligand ZINC00899870 is actively involved in the binding with all the four DENV serotypes.

BLI based binding interactions

A comparative analysis of the interaction revealed that RA binds to EDIII-DENV1 with the highest affinity (K_{off} value of 1.05 s^{-1}) owing to its slowest dissociation rate (K_{off}) among all four serotypes; to EDIII-DENV2 with ten times lower affinity (K_{off} value of 5.63 x 10^{-01} s^{-1}); to EDIII-DENV3 with an affinity of (K_{off} value of 4.63 x 10^{-02} s^{-1}); and to EDIII-DENV4 with lowest affinity (K_{off} value of 3.53 x 10^{-02} s^{-1}) due to its fastest dissociation rate. Binding affinity can be arranged in increasing order of magnitude as EDIII-DENV1 > EDIII-DENV2 > EDIII-DENV3 > EDIII-DENV4.

Cytotoxicity of RA

Cytotoxicity of RA was determined on Vero cells by a MTT assay. The median cytotoxicity (CC_{50}) of RA was determined to be 34.67 mg/mL (Figure 3) on Vero cells.

Virus titer

SEROTYPE	TITER (log_{10} PFU/ML)
DENV 1	6.308
DENV 2	7.091
DENV 3	6.682
DENV 4	7.246

DENV serotypes titre in (log_{10} PFU/mL) as determined by plaque assay

Antiviral activity

The EC_{50} for RA in inhibiting DENV1 was 13.73 µg/mL (SI = 2525), for DENV2 was 77.74 µg/mL (SI = 446), for DENV3 was 244 µg/mL (SI = 142) and for DENV4 was 280 µg/mL (SI = 124). The effect of RA was variable on different serotypes, indicating highest effect on serotype DENV1 and DENV2 followed by DENV3 and least on DENV4. The effect of RA was variable on different serotypes, indicating highest effect on serotype DENV1 and DENV2 followed by DENV3 and least on DENV4.

Selectivity Index of the drug (RA) was calculated using SI= CC_{50}/IC_{50}

Serotype	**IC_{50} (µg/mL)**	**CC_{50} (mg/mL) on Vero cells**	**SI value**
DENV1	13.73	34.67	2525
DENV2	77.74		446
DENV3	224		142
DENV4	280		124

SI (selectivity index) value calculated by using formula SI=CC_{50}/IC_{50}

DISCUSSION

In the present study we began with exploring phytochemicals with a likely antiviral activity against DENV which presents unique challenges in the conventional drug discovery approach due to the fact that dengue is caused by four serotypes that are genetically related being antigenically distinct. Also, the fact that severity of infection varies with primary and secondary infection. The phytochemical under consideration should present a wide spectrum effect against the maximum number of serotypes to qualify it as an anti-dengue viral agent. From literature survey we identified two phytochemicals that demonstrated a promising candidature. Lutein and RA both are well known and established for their immunomodulatory and antioxidant properties along with antiviral activities.

The first approach was to evaluate the virtual binding ability of Lutein and RA towards the EDIII of four DENV serotypes by using bioinformatics tools in an *in silico* study. The DENV envelope domain III (EDIII) protein is recognized to be involved in the host cell adhesion and hence plays a pivotal role in viral infection. Therefore, blocking EDIII could theoretically restrict the virus from receptor recognition, thereby limiting viral propagation. The structures of EDIII proteins of DENV retrieved from PDB with PDB ID: 3IRC, 2JSF, 3VTT and 2H0P corresponding to EDIII of DENV-1, DENV-2, DENV-3, and DENV-4 respectively. Sequence analysis and structural comparison between EDIII of four serotypes using CLUSTALW software and PyMOL software respectively demonstrated that the four serotypes possess considerable similarity between them indicating around 75 % of conserved regions.

Four ligands for RA and four for Lutein were obtained from ZINC database based on the availability in database. The ligands were screened for pharmacological properties using Lipinski's rule of five and oral bioavailability, using a software package MOLSOFT LLC. It was

predicted that Lutein ligands did not clear few parameters of the Lipinski's filter, such as molecular weight must be <500 Da, Log P <5, refractive molar range must be between 40-130 with a drug likeliness score of -0.33. Whereas, RA ligands cleared the filter test successfully with a drug likeliness score of 0.37.

A major prerequisite for an antiviral drug candidate is the safety evaluation. Hence, both the ligands were tested for mutagenic and/or non-carcinogenic properties using LAZAR database and established to be non-carcinogenic.

Using computational molecular docking analysis and ligand interaction study with the four receptors, we determined the related binding energy of interaction between the EDIII of the four DENV serotypes and 8 compounds of Lutein and RA (4 for Lutein and 4 for RA) by AutoDockVina and ligand interaction study using Molecular Operating Environment (MOE) software. RA ligand ZINC899870 established wide spectrum effect demonstrating significant binding ability with a low binding energy against all serotypes of DENV, being most effective against DENV 1, DENV 2 and DENV4 in the increasing order of magnitude. On the other hand, Lutein was most effective in binding ability having very low binding energy with DENV 3. RA established superior binding dynamics against all four serotypes. From the results predicted by MOE, hydroxyl group of the ligands play an important role in the interaction of the ligands to the proteins. With the evidence of the findings, hydroxyl (OH) of 3, 4-dihydroxyphenyl group of RA ligand ZINC899870 is actively involved in the binding with all the four DENV serotypes. Therefore, 3, 4-dihydroxyphenyl can be further investigated as a potential pharmacophore in the discovery of antiviral chemotherapeutic agents against DENV across all the four serotypes. Leading credence to the investigations, it was decided that only RA shall be considered for the *in*

vitro studies moving forward as Lutein has failed the Lipinski filter and the fact that RA demonstrated a considerable proficiency as a therapeutic candidate in comparison.

For RA to be an effective antiviral agent, it should bind to its target first. In this investigation, we demonstrate for the first time, real-time label-free kinetics of interaction between a small molecule compound, RA and the EDIII protein of the four DENV serotypes using a BLI based platform. The dissociation rates (K_{off}) of RA to the EDIII proteins demonstrate interaction which can be arranged in increasing order of magnitude EDIII-DENV1 > EDIII-DENV2 > EDIII-DENV3 > EDIII-DENV4.

A major prerequisite for an antiviral drug candidate is the safety evaluation; hence the cytotoxicity of RA was evaluated in cell culture. The CC_{50} for the same was evaluated to be 34.67 mg/mL on Vero cells *in vitro* by MTT assay. The potential *in vitro* antiviral activity of RA against the four DENV serotypes was evaluated using the plaque assay method on Vero cell line. The plaque assay was employed for virus titration of the stock and also for the antiviral assay by virtue of reduction in the number of plaques.

The antiviral activity of RA (EC_{50}) can be arranged in an increasing order of magnitude EDIII-DENV1 > EDIII-DENV2 > EDIII-DENV3 > EDIII-DENV4. These findings suggest that RA does exhibit antiviral activity against all the four serotypes of DENV. However, the effect of RA was variable among different serotypes, demonstrating highest effect on serotype DENV1 and DENV2 followed by DENV3 and DENV4. Also, the plaque assay results were in good agreement with the BLI based binding study. This study further enlightens the malleability of RA to interact with all four serotypes of DENV and interfere in the virus pathogenesis. Further, *in vivo* analysis of RA as a potential anti-dengue viral candidate needs to be evaluated to validate the observations of our present study.

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